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(21) International Application Number: PCT/EP99/05489 (22) International Filing Date: 27 July 1999 (27.07.99) (30) Priority Data: 98202524.9 28 July 1998 (28.07.98) EP (71) Applicant (for all designated States except US): VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECH- NOLOGIE VZW [BE/BE]; Rijvisschestraat 120, B-9052 Zwijnaarde (BE). (72) Inventors; and (75) Inventors/Applicants (for US only): BROEKAERT, Daniël [BE/BE]; Hovigen 5, B- 9950 Waarschoot (BE). VANDEK- ERCKHOVE, Joël, Stefaan [BE/BE]; Rode Beukendreef 27, B-8210 Loppem (BE). VERHEE, Annick [BE/BE]; Pas- toor Denyslaan 60, B-8810 Lichtervelde (BE). WAELPUT, Wim [BE/BE]; Nieuwerkerkenstraat 111, B-9100 Nieuw- erkerken-Waas (BE). TAVERNIER, Jan [BE/BE]; Bottel- weg 2, B-9860 Balegem (BE). (74) Common Representative: VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE VZW; Rijviss- chstraat 120, B-9052 Zwijnaarde (BE).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: LEPTIN-MEDIATED GENE-INDUCTION (57) Abstract Using the PC12 cell line as a model system a series of transcripts induced through activation of the leptin receptor or gp130 was identified. Based on kinetic studies on undifferentiated PC12 cells, two distinct gene-sets could be discerned: STAT-3, SOCS-3, Metallothionein-II, the serine/threonine kinase Fnk and the rat homologue of MRF-1 which are immediate early response genes, and Pancreatitis Associated Protein I, Squalene Epoxidase, Uridinediphosphate Glucuronyl Transferase and Annexin VIII, which are late induced target genes. In the latter case only, a strong co-stimulation with the adenylate cyclase activator forskolin was observed. Two additional transcripts encoding Leptin Induced Protein I (LIP-I) and Leptin Induced Protein II (LIP-II) were also identified. LIP-II is a rat orthologue of the human Down Syndrome Cell Adhesion Molecule (DS-CAM). In both cases, no forskolin co-stimulatory effect was observed. On PC12 cells differentiated to a neural phenotype by combined β -NGF and forskolin treatment, Pancreatitis Associated Protein III, Peripherin and Mx2 protein were further identified as being regulated by leptin. Finally, from an RDA experiment using mRNA from either hyper-IL-6- or leptin-induced PC12 cells, the Reg gene, another member of the Pancreatitis Associated Protein family, and HIP-1 were identified as selectively up-regulated by H-IL-6. STAT-3 and SOCS-3 have been recognized in leptin signalling <i>in vivo</i> before. In this invention it is also demonstrated that leptin modulates the <i>in vivo</i> expression of the MT-II, Fnk and Pancreatitis Associated Protein I genes.		

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Leptin-mediated gene-induction

5 The current invention relates to the use of leptin and/or a cytokine which binds to a receptor complex comprising gp130, optionally in combination with a compound acting on adenylate cyclase or one of its downstream targets, to activate a signalling cascade whereby as a result thereof immediate early response and/or late target genes are induced in neuro-endocrine cells or in cells from neuro-endocrine origin.

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Background information for the invention

15 In healthy conditions, assimilation, storage and utilisation of nutrient energy constitute a highly integrated homeostatic system. Maintaining a relatively constant level of energy stores, and hence body weight, requires the achievement of a balance between food intake and energy expenditure. The "set point" hypothesis proposes a co-ordinated regulation by control centres in the central nervous system. Hypothalamic nuclei are believed to be the sites at which the "set point" is regulated, given their important role in installing homeostasis through regulation of food intake (hunger versus satiety), body weight, energy expenditure (adaptive thermogenesis) and hormone integration that steers substrate inter-conversion, storage and mobilisation in due times.

20 An expanding array of (neuro)peptides and neurotransmitters that alter food intake when administered peripherally or directly into the hypothalamus is being discovered. Mouse models of obesity include five apparently single gene mutations. The most intensively studied mutations occur in the ob and db genes, which are intimately involved in the control of body fat deposits. This control involves a signalling pathway including the action of a hormone secreted by adipocytes and acting through specific receptors in areas of the brain that govern ingestive

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behaviour and metabolic activity (Flier, 1997; Flier and Maratos-Flier, 1998; Spiegelman and Flier, 1996).

Cloning of the mouse ob gene and its human homologue led to the identification of the ob gene product (ob protein) now named leptin (Zhang et al., 1994). It is secreted primarily by white adipocyte tissue as a non-glycosylated 146-amino acid polypeptide with a MW of 16 kDa. Consistent with its role as the postulated adipose-derived satiety factor, administration of recombinant leptin to ob/ob mice caused rapid reversal of the obese phenotype (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995) by decreasing food intake and increasing energy expenditure. Furthermore, administration of leptin corrects most, if not all of the metabolic and endocrine defects, including sterility, in the ob/ob mice (Chehab et al., 1996).

Leptin displays no apparent sequence similarity to any other known protein. However, based on structure prediction analysis, leptin is identified as a member of the haematopoietic cytokine receptor family carrying the typical four α -helical bundle structure. Accordingly, its receptor belongs to the class 1 cytokine receptor superfamily. As a cytokine, leptin has a pleiotropic role including effects on the haematopoietic system, in immune defence and inflammation, reproduction and pregnancy, regulation of renal function and acceleration of the onset of puberty in female rodents. In addition to its central hypothalamic action, leptin may also exert effects on extraneural tissues extending its metabolic effects. This includes repression of fatty acid and lipid synthesis, triacylglyceride depletion of tissues and increased expression of enzymes of fatty acid metabolism and of the uncoupling protein UCP-2, believed to play an important role in thermogenesis. Recent studies show that leptin also plays a role in hematopoiesis, in fatty acid homeostasis in cells, in hepatic metabolism and in the protection against TNF-induced toxicity. Leptin also stimulates the proliferation of CD4⁺ T-cells and has direct effects on endothelial cells, and cells of the gastro-intestinal tract.

Leptin exerts its effects upon binding to a high affinity receptor, the product of the db gene. This receptor was originally cloned from mouse choroid plexus and may exist in at least six isoforms through alternative splicing (Tartaglia et al., 1995; Lee et

al., 1996). So far, most attention has been focused on a ubiquitously expressed splice variant with short cytoplasmic tail (OB-Ra), and on the isoform with a long cytoplasmic domain (OB-Rb), which is predominantly expressed in certain nuclei in the hypothalamus. RT-PCR and Northern blot analysis also showed expression of the latter isoform in peripheral tissues including pancreas, liver, lung, kidney and adrenals, intestine and adipose tissues, as well as in endothelial cells and CD4⁺ helper T-cells. The detailed structure of the murine leptin receptor gene was recently described. In the db/db mice, a point mutation causing the use of a cryptic splice site leads to the expression of a receptor with a truncated cytoplasmic tail, which is likely signalling deficient. The fatty (fa) gene in rats appears to be a functional homologue of the mouse db gene, due to a Gln269Pro substitution in the extracellular domain of the receptor, leading to impaired signalling and obesity. Underscoring the evolutionary conserved role of this pathway, homologous mutations were recently described in humans. Severe early-onset obesity was observed in patients with mutations leading to either impaired leptin (Montague et al., 1997) or leptin receptor (Clement et al., 1998) function.

Based on sequence homology and functional aspects, the leptin receptor is considered a member of the Class I cytokine receptor superfamily, and is most closely related to gp130, the signalling component of the IL-6R complex, and to the LIF and G-CSF receptors. As such, the leptin receptor contains typical motifs involved in signalling such as a JAK tyrosine kinase binding site, and a Box 3 involved in recruitment of STAT-3 upon ligand-induced receptor tyrosine phosphorylation. The long isoform is generally believed to be the signalling competent receptor although divergent signalling capacities have been described for the long and short isoforms, which both contain the Box 1 motif. The receptor functions as a (ligand-induced) homodimer, independent of gp130. Leptin binding leads to the activation of JAK2 and multiple STATs (STAT-1, STAT-3 and STAT-5b), however only STAT-3 activation was observed in hypothalamic centres *in vivo*. In established hepatoma cell lines stably expressing the OB-R long isoform, the leptin receptor appears to be functionally equivalent to the endogenous IL-6R.

One target for leptin action in the hypothalamus is neuropeptide Y (NPY), a key effector of nutritional homeostasis that stimulates appetite. Leptin induces inhibition of NPY biosynthesis and release. The observation that NPY-deficient mice did not completely reverse the obesity, although NPY is required for full manifestation of the ob/ob phenotype, makes it likely that other leptin targets must exist. Such alternative candidate targets include glucagon-like peptide 1 (GLP-1) produced in the brain stem and causing reduced food intake, the melanin-concentrating hormone also involved in hypothalamic regulation of ingestive behaviour, the hypothalamic corticotropin-releasing factor (CRF) inhibiting appetite and stimulating metabolism, and the recently described orexins and cocaine- and amphetamine-regulated transcript (CART), a hypothalamic satiety factor. Effects on the expression of the pro-opio-melanocortin gene by leptin have also been described.

Obese humans, except patients carrying the rare, above mentioned mutations in the genes for leptin or its receptor, generally produce higher levels of circulating leptin, suggesting that obesity is associated with "leptin resistance". Such resistance could conceivably occur at several levels: peripheral leptin dysfunction, dysregulation of the saturable leptin transport through the blood brain barrier, and also the expression of and signalling by the hypothalamic leptin receptor. Variable mechanisms leading to leptin resistance are suggested by studies in murine models (Halaas et al., 1997).

Detailed description of the invention

To gain more insight in leptin signalling and function, the analysis of leptin-mediated gene induction and signalling in the neuro-endocrine-type PC12 cell line was started and both immediate early response and late target gene sets were identified. The PC12 cell line appears to be a physiologically relevant cell line to study leptin-induced gene regulation, since three of the identified genes have been implicated in leptin function or obesity *in vivo*. Activation of STAT-3 protein, and up-regulation of SOCS-3 gene expression have been shown to occur in hypothalamic

nuclei by leptin treatment of ob/ob, but not of db/db mice (Vaisse et al., 1996; Bjorbaek et al., 1998). The identification of metallothionein-II as a leptin-induced immediate early response gene is of special interest since it was recently shown that mice with targeted disruption of both MT-I and MT-II genes become obese, with elevated plasma leptin levels. Sofar, no other clear physiological role could be attributed to these proteins. According to the invention it is further demonstrated that leptin regulates the expression of MT-II *in vivo*. In addition leptin-mediated regulation *in vivo* of the serine/threonine kinase Fnk, and of the Pancreatitis Associated Protein I is shown. Both gene transcripts were identified as leptin-induced in PC12 cells, further underscoring the validity of this *in vitro* model system. Furthermore new leptin up-regulated transcripts were identified in PC12 cells differentiated towards a neuronal phenotype, as well as transcripts induced via triggering of the gp130 signaling component of receptors for the interleukin 6 family of cytokines.

It should be clear that molecules acting downstream of the leptin receptor and modulating leptin function offer potential use in treatment of human obesity or other metabolic disorders including anorexia.

It is our invention that leptin and/or a cytokine which binds to a receptor complex comprising gp130, optionally in combination with a compound acting on adenylate cyclase or acting directly or indirectly on one or more of the downstream targets of said cyclase, can be used to activate a signalling cascade whereby as a result thereof immediate early response and/or late target genes are induced in neuro-endocrine cells or cells of neuro-endocrine origin.

Said signalling cascade is preferably activated through a leptin receptor while the neuro-endocrine cells are PC12 cells.

To the invention also belongs the use of leptin and/or a cytokine which binds to a receptor complex comprising gp130, optionally in combination with said compound wherein the compound is forskolin.

In addition, PC12 cells may be differentiated to a neuronal state upon treatment with β -NGF (beta-nerve growth factor) and forskolin, prior to activation of the said receptor complexes.

The use of leptin according to the invention, optionally in combination with forskolin, provides an induction of immediate early response genes such as STAT-3, SOCS-3, Metallothionein-II, the serine/threonine kinase Fnk and of MRF-1, while the combination of leptin and forskolin has a pronounced induced effect on late target genes such as Pancreatitis Associated Protein I, Squalene Epoxidase, Uridinediphosphate Glucuronyl Transferase and Annexin VIII.

Two additional transcripts encoding Leptin Induced Protein I (LIP-I) and Leptin Induced Protein II (LIP-II) were also identified. LIP-II is a rat orthologue of the human Down Syndrome Cell Adhesion Molecule (DS-CAM). In both cases, no forskolin co-stimulatory effect was observed. On PC12 cells differentiated to a neural phenotype by combined β -NGF and forskolin treatment, Pancreatitis Associated Protein III, Peripherin and Mx2 protein were further identified as being regulated by leptin. Finally, from an RDA experiment to search for transcripts differentially induced by hyper-IL-6 compared to leptin in PC12 cells, the Reg gene, another member of the Pancreatitis Associated Protein family, and HIP-1 were identified as selectively up-regulated by H-IL-6.

To the scope of the invention also belongs a method of screening for molecules in mammalian cells, in particular human cells using human homologues of the genes isolated according to the current invention, which interfere directly or indirectly with the induction of immediate early response genes and/or late target genes or with the activity of the products of said genes; said genes are inducible by leptin and/or a cytokine which binds to a receptor complex comprising gp130, optionally in combination with a compound acting on adenylate cyclase or acting directly or indirectly on one or more of its downstream targets.

Part of the invention are thus obtainable molecules by the screening method and a pharmaceutical composition comprising said molecule or molecules.

As used herein, the term "composition" refers to any composition such as a pharmaceutical composition comprising as an active ingredient said molecule or molecules according to the present invention possibly in the presence of suitable excipients known to the skilled man and may thus be administered in the form of any

suitable composition as detailed below by any suitable method of administration within the knowledge of a skilled man. The preferred route of administration is parenterally. In parenteral administration, the compositions of this invention will be formulated in a unit dosage injectable form such as a solution, suspension or emulsion, in association with a pharmaceutically acceptable excipient. Such excipients are inherently nontoxic and nontherapeutic. Examples of such excipients are saline, Ringer's solution, dextrose solution and Hank's solution. Nonaqueous excipients such as fixed oils and ethyl oleate may also be used. A preferred excipient is 5% dextrose in saline. The excipient may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, including buffers and preservatives.

The isolated functional molecules of the invention thus obtained are administered at a concentration that is therapeutically effective to prevent allograft rejection, GVHD, allergy and autoimmune diseases. The dosage and mode of administration will depend on the individual. Generally, the compositions are administered so that the isolated functional protein/molecule(s) is given at a dose between 1 $\mu\text{g/kg}$ and 10 mg/kg, more preferably between 10 $\mu\text{g/kg}$ and 5 mg/kg, most preferably between 0.1 and 2 mg/kg. Preferably, it is given as a bolus dose. Continuous short time infusion (during 30 minutes) may also be used. The compositions comprising the isolated functional protein/molecule(s) according to the invention may be infused at a dose between 5 and 20 $\mu\text{g/kg/minute}$, more preferably between 7 and 15 $\mu\text{g/kg/minute}$.

According to a specific case, the "therapeutically effective amount" of the isolated functional protein according to the invention needed should be determined as being the amount sufficient to cure the patient in need of treatment or at least to partially arrest the disease and its complications. Amounts effective for such use will depend on the severity of the disease and the general state of the patient's health. Single or multiple administrations may be required depending on the dosage and frequency as required and tolerated by the patient.

In the context of this description the terms "molecules", "proteins" and "compounds" are interchangeable unless mentioned to the contrary.

In order to further disclose the current invention a more detailed explanation is given hereunder.

Synergistic effects of leptin and forskolin or β -NGF on PC12 cells

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In order to study leptin receptor signalling in a neuro-endocrine-related cell type, we transiently transfected PC12 cells with expression vectors for the long or the short isoform of the mouse leptin receptor (pMET7-mLRlo and pMET7-mLRsh respectively) and monitored gene induction by leptin. The PC12 cell line was
10 established from a transplantable rat adrenal pheochromocytoma and is frequently used as a model system for differentiation of neuronal cells. Stimulation with recombinant rat β -nerve growth factor (β -NGF) leads to a growth arrest and the formation of dendritic processes and expression of neuronal markers. Binding studies using a mouse leptin-SEAP fusion protein, and RT-PCR analysis showed
15 that neither undifferentiated nor differentiated PC12 cells express leptin receptors. To determine leptin responsiveness, different reporter gene constructs were developed, based on the observation that stimulation of the leptin receptor leads to changes in the expression of a variety of neuropeptides, including NPY and POMC. A first reporter construct contains a 500bp fragment of the rat neuropeptide Y (rNPY)
20 promoter sequence coupled to the luciferase gene (pGL3-rNPYluc). Fig. 1A shows that leptin stimulation of PC12 cells co-transfected with the rNPY reporter construct and pMET7-mLRlo but not with pMET7-LRsh led to a moderate stimulation of luciferase activity. However, co-stimulation in the former case with forskolin, a stimulator of adenylate cyclase, showed an up to 14-fold enhanced reporter activity.
25 Optimal co-stimulatory conditions were determined as 100 ng/ml leptin and 10 μ M forskolin (Fig. 1B). This effect was optimal approximately 72 hours post stimulation.

Leptin responsiveness in PC12 cells was further investigated using a clone stably expressing the long isoform of the mouse leptin receptor (PC12-LR8, see below). After transfection of PC12-LR8 with a reporter construct based on the
30 human POMC (proopiomelanocortin) promoter (pGL3-POMCluc) (Fig. 1C), or a reporter construct based on the rat Pancreatitis Associated Protein I promoter (see

below) (Fig. 1D), leptin induced luciferase activity was measured. Administration of β -NGF (1 ng/ml) mimicked for both reporter constructs the co-stimulatory action of forskolin. The β -NGF and forskolin effects appeared to be additive in this clone (Fig. 1C and 1D).

5

Identification of genes regulated by leptin in PC12 cells.

To search for genes regulated by leptin in the PC12 cell line, a RDA (representational difference analysis) experiment was performed using a modification
10 of the original method (Hubank and Schatz, 1994). Using this procedure it is possible to clone amplicons corresponding to transcripts from leptin-forskolin co-stimulated PC12 cells, transiently transfected with pMET7-mLRlo. After three rounds of subtraction/amplification, selectively amplified bands were purified and subcloned in the pCDNA3 or pCR-Blunt vector (Invitrogen). Subsequent DNA sequencing
15 revealed that a strongly induced transcript encoded the rat Pancreatitis Associated Protein I (rPAP I). Based on this observation, a simple one-tube RT-PCR based procedure was set up to select for PC12 subclones, stably expressing the leptin receptor long isoform (Fig. 2A). One stable clone, PC12-LR8, was chosen for further experiments (Fig. 2B). Individual inserts from the cloned amplicon collection were
20 radiolabelled and leptin-dependent gene regulation was verified and studied in more detail by Northern blot analysis on the PC12-LR8 cell line. A total of 11 leptin-regulated genes were identified, as shown in Table 1. Only up-regulated genes were observed; a parallel experiment selecting for leptin-induced down-modulation of gene expression did not yield any amplicons. Interestingly, several of the identified gene
25 products have already been implicated in leptin signalling or obesity.

Annexin VIII is a calcium-dependent phospholipid-binding protein expressed in lung, skin, liver, and kidney. The physiologic function of annexin VIII remains unknown.

FGF-inducible kinase (Fnk) was first identified as a serine/threonine kinase
30 induced by Fibroblast Growth factor FGF-1 in murine NIH 3T3 fibroblasts. It is closely related to the polo-family of serine/threonine protein kinases (including human Prk,

mouse Snk, human and murine Plk, mouse Sak, Drosophila Polo, and yeast Cdc5). In adult animals, Fnk-mRNA is expressed at high levels in skin, but is also detected in brain, intestine, kidney, lung and ovary. In newborn animals, Fnk transcripts are expressed in high levels in intestine, kidney, liver, lung and skin. The related Prk and Plk kinases are induced by cytokines in hematopoietic cells (Li et al., 1996) and in primary T-cells (Holtrich et al., 1994) respectively. These kinases may play a role in cell proliferation, but their precise role remains unclear.

Metallothionein-II (MT-II) is a member of a family of metal-binding proteins that are reported to function in the detoxification and homeostasis of heavy metals, in the scavenging of free radicals and in the acute phase response. Importantly, it was recently reported that MT-I/II deficient mice on a C57BL/6J-129Ola genetic background show mild, late onset obesity (Beattie et al., 1998).

Modulator Recognition Factor 1 (MRF-1) is a DNA binding protein belonging to a poorly characterised protein family. (GenBank accession number for sequences of the human homologue and the related human MRF-2 are M62324 and M73837, respectively).

Pancreatitis Associated Protein I (PAP I) is a C-type lectin-related secretory protein present in small amounts in the rat pancreas (in both endocrine and exocrine cells) and is rapidly overexpressed during the acute phase of pancreatitis. The physiological role of PAP I is still unclear at present, but its involvement in acute pancreatitis as an acute phase protein suggests a role in tissue protection and/or recovery. PAP I is also expressed in normal intestine and is induced by feeding (Duseti et al., 1995).

Signal transducer and activator of transcription 3 (STAT-3) is a key transcription factor mediating the signals for a variety of cytokines. A critical role for STAT-3 in leptin signalling has been reported in cell lines (Baumann et al., 1996) and in ob/ob mice (Vaisse et al., 1996).

Squalene epoxidase is a rate-limiting enzyme in cholesterol biosynthesis. Transcriptional regulation of squalene epoxidase by sterol is part of a co-ordinately controlled biosynthetic pathway (Nakamura et al., 1996).

Suppressor of Cytokine Signalling-3 (SOCS-3) belongs to a growing family of SOCS proteins. These proteins act as intracellular inhibitors of several cytokine signal transduction pathways. It was recently reported that SOCS-3 may contribute to leptin resistance *in vivo*. (Bjorbaek et al., 1998).

5 Uridinediphosphate Glucuronyl Transferase (UGT) is a key enzyme involved in bilirubine and drug detoxification, as well as in steroid inactivation and excretion, and in proteoglycan side chain formation. Conjugation of compounds with glucuronic acid renders the molecule strongly acidic and more water soluble at physiological pH than the precursor molecule thereby facilitating metabolism, transport and secretion.

10 Two amplicons were cloned respectively derived from transcripts from sofar unidentified genes coding for Leptin Induced Proteins LIP-I and LIP-II (Fig.3,4). LIP-II belongs to the immunoglobulin superfamily and is a rat orthologue of the human Down Syndrome Cell Adhesion Molecule, DSCAM. Expression of DSCAM occurs primarily in the brain, and has been implicated in neural development.

15 These Leptin Induced Proteins LIP-I and LIP-II are hitherto unknown and are therefore new identified nucleic acid/protein sequences as such and thus form part of the current invention.

20 The search for leptin-regulated genes was also extended to differentiated PC12 cells (Fig. 5). Adherent PC12-LR8 cells were treated with β -NGF and forskolin for 5 days, which led to a growth arrest, the formation of branched neuritic processes and the accumulation of small vesicles. Again, an RDA experiment was performed using mRNAs from differentiated cells treated with leptin for 24 hours, or from untreated cells, both in the continued presence of β -NGF and forskolin. Three leptin
25 up-regulated transcripts were identified (Table 1). Interestingly, one of the gene products, PAP III, belongs to the same protein family as PAP I.

Mx2 is a type I interferon-inducible gene, involved in antiviral defence. High expression levels are observed in differentiated PC12 cells, in contrast to very weak expression in undifferentiated cells.

30 Peripherin is a cytoskeletal component, which is part of the type III intermediate filament. Increased expression levels are observed in differentiated

cells when compared to undifferentiated cells. Up-regulation has been described by Interleukin 6 (IL-6) and Leukemia inhibitory Factor (LIF).

Pancreatitis Associated Protein III (PAP III) is a member of the PAP family of C-type animal lectins, mentioned above. It is also induced in normal intestine upon feeding (Dusetti et al., 1995).

Another RDA experiment was performed to identify transcripts differentially (and selectively) induced by hyper-IL-6 (H-IL-6), compared to leptin (Fig 6). H-IL-6 is a fusion protein of IL-6 and the secreted IL-6R subunit (Fischer et al., 1997). In most cases, H-IL-6 treatment led to up-regulation of the same gene-set as observed with leptin. Two H-IL-6 induced transcripts, not induced by leptin, were identified. HIP-I (Hyper-IL-6 induced protein I) corresponds to a novel gene transcript (Fig. 7). Reg is another member of the PAP family of C-type lectins. Reg was originally isolated from a cDNA library from regenerating rat pancreas islets. Other names are Pancreatic Stone Protein (PSP), Pancreatic Thread Protein (PTP), Islet Cell Regenerating Factor and Lithostatin. It is considered as a growth factor for pancreatic beta cells. Similarly, the previously identified, related transcripts encoding PAP I and PAP III, also appear to be strongly induced by H-IL-6, in contrast to leptin, which requires forskolin co-stimulation.

Kinetics of induction identifies immediate early response genes and late target genes.

Next, the kinetics of induction of the above-mentioned transcripts in non-differentiated PC12 cells was analysed upon leptin treatment. Interestingly, two types of gene-sets could be distinguished: a group of immediate early response genes, including Fnk, MT-II, MRF-1, STAT-3 and SOCS-3, in which case induction occurs within 4 hours (Fig. 8A), and a series of late activated target genes including PAP I, UGT, Ann VIII and squalene epoxidase, with induction not before 6 hours after stimulus (Fig. 8C). Next the induction of the immediate early response genes was investigated in more detail (Fig. 8B): Optimal stimulation varied between 30 minutes

(SOCS-3) and 8 hours (STAT-3) post induction. Kinetics of synthesis of SOCS-3 mRNA showed a rapid decline already 2 hours post stimulation. In case of the late target gene-set, optimal mRNA levels were observed between 22 hours (PAP I, UGT) and over 96 hours (Annexin VIII, Squalene epoxidase) post induction.

5 As is apparent from Fig. 8, the forskolin co-stimulation also allows distinguishing both gene-sets. In case of the immediate early response genes, some co-stimulation is apparent for MT-II and MRF-1 but only at later time points, and not in the early induction phase. In case of SOCS-3, forskolin co-treatment even leads to a reduced induction. In contrast, a strong co-stimulatory effect is seen in case of PAP
10 I, UGT, Ann VIII and squalene epoxidase from 22 hours post stimulation.

To address the mechanism of induction of the late gene set, the effect of the protein synthesis inhibitor cycloheximide on rPAP I and Annexin VIII mRNA expression was measured. Treatment with cycloheximide (50 μ M, starting at 30 minutes before induction for 8.5 hours) showed a strongly reduced expression 24
15 hours post induction, implying that de novo protein synthesis is required for induction of the late target gene set.

Regulation of MT-II, Fnk and PAP I expression by leptin in ob/ob mice.

20 In order to assess the value of our *in vitro* model system for obesity, we investigated the regulation by leptin of a subset of the identified genes *in vivo*. Recombinant human leptin (R&D Systems) was administered intraperitoneally to leptin deficient ob/ob mice in a single dose of 100 μ g leptin /mouse. Mice were killed by cervical dislocation 5 hours after treatment and total RNA was isolated from liver
25 and jejunum. Northern blot analysis was performed using respectively MT-II, Fnk and PAP I as probe (Fig. 9). Leptin treatment of ob/ob mice caused a clear induction of MT-II and Fnk mRNA expression in liver, while expression of PAP I in jejunum was downregulated by leptin. In a separate experiment 3 out of 4 ob/ob mice showed clear induction of MT-II and Fnk mRNA in liver two hours after stimulation with leptin
30 (100 μ g/mouse) in combination with the 2A5 antibody (200 μ g/mouse). 2A5 has been shown before to potentiate leptin activity *in vivo* (Verploegen et al., 1997). Twelve

hours after injection, expression levels returned to control levels.

Effects of starvation on MT-II, Fnk and PAP I expression in wild type mice.

5 We also investigated the effect of starvation on MT-II and Fnk expression in liver of wild type mice (Fig. 10A). Mice, starved for 24 hours, received a single injection of human leptin intraperitoneally (R&D Systems, 50 µg/mouse) in combination with the 2A5 anti-human leptin antibody (200 µg/mouse). As a control, a single injection with endotoxin free PBS was performed similarly. The leptin effect
10 was evaluated by Northern blot analysis after 2, 6 and 12 hours in prolonged starvation conditions. Starvation conditions led to a moderate increase in MT-II and Fnk mRNA expression in the liver. This effect was markedly enhanced by leptin plus 2A5 treatment, leading to a strong induction of MT-II and Fnk expression two hours post injection. Six hours after leptin administration MT-II mRNA expression returned
15 to the level observed in the PBS treated control mice, whereas Fnk expression was maintained at higher expression level, compared to the control group. Starvation also led to a spontaneous induction of MT-II mRNA in jejunum. In contrast with the observation in liver, this effect was suppressed by leptin + 2A5 treatment 6 hours post injection. The expression levels of MT-II recovered to control levels 12 hours
20 post injection (Fig. 10B). A similar pattern was observed for PAP I mRNA expression in jejunum, showing a reduction 24 hours after leptin + 2A5 treatment in starved mice, compared to the PBS treated controls.

25

In order to further explain the invention some examples are given hereunder for the sake of clarity.

30

Examples

5 Example 1

Cell culture and transfection.

PC12 cells were cultured in RPMI 1640 medium with glutamax-I (GibcoBRL) containing 10% heat-inactivated foetal calf serum (iFCS) and gentamycin (50 µg/ml). The cells were treated with medium alone or supplemented with 100 ng/ml of mouse
10 leptin (R&D Systems), with forskolin (Sigma) at a concentration of 10 µM or with a combination both, unless otherwise indicated.

For neuronal differentiation, resuspended PC12 were seeded on rat tail collagen (Collaborative Biomedical Products) coated plates at 2-3 10^6 cells/25 cm² flask in RPMI 1640 medium with glutamax-I containing 10% heat-inactivated horse serum,
15 5% iFCS and gentamycin. After one day of culturing, the non-adherent cell fraction was removed by refreshing the medium. Differentiation was induced by a combined β-NGF(10 ng/ml, R&D Systems) and forskolin (10 µM) treatment for approximately 5 days. Medium was replaced after 2-3 and 5 days.

The pMet7 vector was used as an expression vector for the long and short isoforms
20 of the mouse leptin receptor (designated pMET7-mLRlo and pMET7-mLRsh, respectively). pMet7 is a modified version of the mammalian pME18S expression vector that utilizes the SRα promoter as described by Takebe (Takebe et al., 1988). PC12 cells were transfected by electroporation using the Equibio "Easyject one" electroporator. Typically, 10^7 cells were electroporated in 0.4 cm electrode gap
25 cuvettes with 5 µg vector at 300V and 1500C. Cell surface expression of each protein was measured by specific binding of the leptin-secreted alkaline phosphatase fusion protein (see below).

Cos1 cells were maintained in DMEM supplemented with 10% iFCS (GibcoBRL), and were transfected with pMET7-leptinSEAP (a vector expressing the mouse leptin-
30 secreted alkaline phosphatase fusion protein) using lipofectamine (Life Technologies). Medium was replaced after 16 h and conditioned medium (CM) was

harvested after 64 h. The estimated concentration of the leptin-SEAP fusion protein was approximately 1 µg/ml.

Example 2

5 **Selection of cell lines stably expressing the long isoform of the leptin receptor.**
PC12 cells were electroporated with the pMET7-mLRlo expression vector together with the pHCMV-MCS vector containing the neomycin resistance marker. Transfected cells were selected for growth in RPMI 1640 medium containing glutamax-I (GibcoBRL) and supplemented with 10% heat-inactivated fetal bovine
10 serum and gentamycin (50 µg/ml). Cells were first grown in selective medium containing 500 µg/ml G418 sulfate (Calbiochem) for seven days and in 750 µg/ml G418 from day eight on. After four weeks of growth, colonies were transferred to 48 well plates in medium containing 750 µg/ml G418. Subclones were selected for leptin responsiveness and PAP I gene activation using a one-tube RT-PCR procedure. In
15 brief, after cell lysis mRNA was hybridised with biotin labelled oligodT and captured to streptavidin-coated tubes. After three times washing, the same tubes were used for the RT-PCR, optimized for detection of PAP I gene induction (mRNA capture and Titan One Tube procedure, Boehringer Mannheim).

20 **Leptin binding assay**

Cell surface expression of leptin receptors on PC12 cells was measured using a mouse leptin-secreted alkaline phosphatase chimeric protein as described (Baumann et al., 1996; Flanagan and Leder, 1990). Briefly, cells were washed 48 h post transfection (wash buffer: RPMI 1640, 0.1% NaN₃, 20 mM Hepes pH7.0, 0.01%
25 Tween 20) and were incubated for 90 min at room temperature with a 1/10 dilution of the Cos1 CM containing the chimeric protein in wash buffer. After 6 successive washing steps, cells were lysed in a buffer containing 1% TX-100, 10 mM Tris. HCl pH7.4 and the lysates were treated at 65°C for 30 min to inactivate endogenous alkaline phosphatases. Alkaline phosphatase activity was measured using the CSPD
30 substrate (PhosphaLight, Tropicx) according to the manufacturers' specifications in a TopCount.NXT Chemiluminescence Counter (Packard).

Example 3

RDA (representational difference analysis).

RDA was used to clone differentially expressed cDNAs from PC12 cells, transiently
5 transfected with the leptin receptor long isoform, or from neuronal differentiated
PC12-LR8 cells, which stably express the leptin receptor long isoform. In both cases,
cloning was performed using mRNA from cells either stimulated with leptin+forskolin
or with forskolin alone. This RDA procedure was essentially performed as originally
described (Hubank and Schatz, 1994) and modified by Braun et al. (Braun et al.,
10 1995). PC12 cells were transfected with the pMET7-LRlo expression vector and
stimulated for 72 hours with forskolin alone or with a combination of forskolin and
leptin. In case of neuronal differentiated PC12-LR8 cells, mRNA was obtained from
cells treated with β -NGF and forskolin as described above for 5 days to induce
neuronal differentiation, followed by a 24h treatment with leptin (100 ng/ml) or
15 without additional treatment. In case of hyper-IL-6 treatment, undifferentiated PC12-
LR8 cells were treated with either H-IL-6 (5 ng/ml) or leptin (100 ng/ml) for 24h, prior
to mRNA isolation and RDA analysis.

mRNAs were isolated using the Fast Track method (Invitrogen). A 2 μ g sample of
mRNA of each cell population was used for RDA analysis. cDNAs were synthesised
20 from the mRNAs and digested with DpnII. Two oligonucleotide adapter molecules,
5'AGCACTCTCCAGCCTCTCACCGCA 3' (R-Bgl-24) and 5'GATCTGCGGTGA 3' (R-
Bgl-12), were ligated to the DpnII-digested cDNA. This mixture was amplified by PCR
with R-Bgl-24 oligonucleotides, and the adapters were excised with DpnII. A second
pair of adapters, 5'ACCGACGTCGACTATCCATGAACA 3' (J-Bgl-24) and
25 5'GATCTGTTCATG 3' (J-Bgl-12) was ligated to the amplified fragments from the
leptin-forskolin stimulated cell population and hybridised with the R-Bgl-24 amplified
cDNA fragments from the forskolin stimulated cell population (R-Bgl adapters
removed) at a ratio of 1:100 for 24h. The hybridisation mix was used as template for
amplification by PCR. A second round of subtraction was performed by removing the
30 J-Bgl adapters from this first round PCR product, ligating a third pair of
oligonucleotide adapters, 5'AGGCAACTGTGCTATCCGAGGGAA 3' (N-Bgl-24) and

5'GATCTTCCCTCG 3' (N-Bgl-12), and hybridised with driver amplicons at a ratio of 1:800. A third round of subtraction and amplification was performed using the same conditions as in the first round. Subsequently the transcripts were subcloned into the pCDNA3 or pCR-Blunt (Invitrogen) vector. Most insert DNAs were sequenced using the Alf Express Sequencer (Pharmacia) with the Autoread Sequencing Kit according to the manufacturer's specifications. Primers for sequencing the inserts were the C15-labeled M13 forward primer for the pCR-Blunt clones, and the 5'-GAACCCACTGCTTAACTGGC forward and 5'-GTCGAGGCTGATCAGCGAGC reverse primers for the pCDNA3 clones. In other cases, sequencing was using an ABI Prism 377 DNA sequencer (Perkin Elmer) using the M13 forward primer.

Example 4

Northern blot and reporter analysis.

15 Total RNA was prepared from PC12 cells using RNeasy method (Qiagen). RNA (10µg) was separated on a 1.5 % agarose, 6% formaldehyde gel, transferred to a nylon membrane (Zeta-Probe GT Genomic, Bio-Rad), and cross-linked using UV radiation. The filters were hybridised for one hour at 68°C in ExpressHyb solution (Clontech) with [³²P]dCTP-labeled DNA-probes and washed 3 times with 2x SSC, 0.05% SDS at room temperature and twice in 0.1x SSC, 0.1% SDS at 50°C. 20 Autoradiographs were obtained by exposing the blots to BioMax MS film (Kodak) with intensifying screens at -70°C. All Northern blots were normalised by hybridisation using a β-actin probe.

Luciferase activity was measured in transfected cells by chemiluminescence. Briefly, 25 1 x 10⁵ cells were lysed in 100 µl of lysis buffer (25 mM Tris, pH 7.8 with H₃PO₄; 2 mM CDTA; 2 mM DTT; 10% glycerol; 1% Triton X-100). 70 µl of luciferase substrate buffer (20 mM Tricine; 1.07 mM (MgCO₃)₄Mg(OH)₂·5H₂O; 2.67 mM MgSO₄; 0.1 mM EDTA; 33.3 mM DTT; 270 µM Coenzyme A (lithium salt); 470 µM Luciferin (Duchefa); 530 µM ATP, final pH 7.8) was added to 100 µl of cell lysate and 30 measured for 5 seconds by TopCount.NXT Chemiluminescence Counter (Packard).

Example 5

Analysis of gene expression *in vivo*

Specific pathogen-free female C57BL/6J-Lep^{ob} mice, 9 weeks old at the beginning of the experiment, further referred to as ob/ob were obtained from The Jackson Laboratory (Maine, USA). Specific pathogen-free C57BL/6NCrlBr mice, 8 weeks old at the beginning of the experiment, further referred to as wildtype (wt) were obtained from Charles Rivers Labs. The animals were housed in a temperature-controlled environment with 12 hour light/dark cycles and received water and food ad libitum, with exception for the starvation experiment. All experiments were performed according to the European Guidelines on Animal Care and Use. Recombinant human leptin (R&D Systems) was diluted in endotoxin free PBS and administered intraperitoneally in a dose of 100 µg/mouse. In case of co-administration of 2A5, a monoclonal antibody raised against human leptin (Verploegen et al., 1997), the dose of leptin was reduced to 50 µg/mouse. The dose of the antibody was 200 µg/mouse. The endotoxin content of the antibody was 0.07 ng/mg protein, as assessed by a chromogenic Limulus amoebocyte lysate assay (Coatest, Chromogenix, Stockholm, Sweden). Animals were sacrificed using cervical dislocation. Tissues were resected immediately and frozen in liquid nitrogen. RNA extraction and Northern blot analysis was performed as described above.

Figure legends

Figure 1. Synergistic signalling of leptin and forskolin in PC12 cells

(A) Leptin-induced NPY promoter activity in transiently transfected PC12 cells. PC12 cells were co-transfected with control vector (columns 1 and 2), pMET7-mLRsh (columns 3 and 4) or pMET7-mLRlo (columns 5 and 6) together with the pGL3-rNPYluc reporter. The different subcultures were mock stimulated (growth medium alone) or treated with mouse leptin (100 ng/ml) for 72 hours. Luciferase activity in cell lysates is shown; data represent the mean ± standard deviation values of assays performed in triplicate.

(B) Forskolin co-stimulation on leptin-induced pGL3-rNPYluciferase activity. PC12

cells were transiently co-transfected with pMET7-mLRlo and the pGL3-rNPYluc reporter. Subcultures were treated with mouse leptin alone (filled squares) or in combination with forskolin at a concentration of 10 μ M (filled triangles). After 72 hours cells were lysed and assayed for luciferase activity. Average values of
5 normalised, relative luciferase activities (x-fold increase) from three independent experiments are shown.

(C, D) Analysis of β -NGF and forskolin co-stimulation on leptin-induced POMC luciferase (C) or rPAP luciferase activity (D). PC12-LR8 cells were transiently
10 transfected with pGL3-hPOMCluc or pGL3-rPAPluc reporter and treated after two days with forskolin (F: 10 μ M), leptin (L: 100 ng/ml), and rat β -NGF (N: 1 ng/ml) as indicated or were left untreated (NI). 24 hours after treatment, cells were lysed and assayed for luciferase activity. Data show the mean value \pm standard deviation of assays performed in fivefold.

15

Figure 2. Selection of a leptin-responsive PC12 subclone.

(A) Expression of rPAP I mRNA in PC12 cells, transiently transfected with the long isoform of the mouse leptin receptor. PC12 cells were transfected with pMET7-mLRlo and treated for 48 hours with medium (NI, non-induced), leptin (L), forskolin
20 (F) or forskolin plus leptin (F+L). Total RNA was prepared and subjected to RT-PCR analysis for rPAP I. Amplified PCR fragments were visualised on a 1% agarose gel. β -actin amplification was used as a control.

(B) Expression of rPAP I mRNA in PC12-LR8 cells.
25 Cells were treated for 48 hours with medium (NI, non induced) or leptin (L). RT-PCR analyses of rPAP I expression. β -actin amplification was used as a control.

Figure 3. Northern blot analysis of LIP-I and LIP-II expression in undifferentiated PC12 cells.

30 PC12-LR8 cells were treated with leptin (L), forskolin (F), or a combination of both (F/L) for the indicated length of time. Hybridisation with the mouse β -actin probe was

used as a control. NI stands for non-induced control cells. Sizes of transcripts are shown on the right. Exposure times to Biomax MS films were 5.5 days for LIP-I and LIP-II, and 1 day for the corresponding β -actin blot, all at -80°C .

5

Figure 4. DNA sequences of cloned amplicons corresponding to LIP-I and LIP-II. Sequences A and B respectively correspond to the cloned amplicons from rat LIP-I and rat LIP-II.

10 **LIP-I (349bp)**

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AGGGCTGCGTCAACACCAAGGGCAGCTACGAGTGTGTGTGCCCACCAGGG  50
AGGAGGCTGCACTGGAATCGGAAGGACTGTGTGGAGATGAGCGGGTGCCT  100
GTCTCGGTCCAAGGCCTCTGCCCAGGCCCAGCTGTCCTGTGGCAAGGTGG  150
GTGGAGTGGAAACTGCTTCCTGTCCTGCCTGGGCCAGAGTCTCTTCATG  200
15 CCGGACTCAGAAACCAGCTACATCCTGAGCTGTGGTGTTCAGGTCTCCA  250
   GGGCAAGGCACCACCGAAGCGCAATGGCACCAGCTCCAGTGTGGGGCCCG  300
   GCTGCTCAGATGCCCCCACCACCCCCATCAGACAGAAGGCCCGCTTCAA  349

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20 **LIP-II (484bp)**

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CGCCTGGACAGAAATGGCTCCCTACACATCTCGCAGACATGGTCAGGGGA  50
CATTGGCACGTATACCTGCCGGGTACTCTCAGCTGGTGGCAATGACTCTC  100
GCAACGCCCACCTGCGAGTCAGGCAGCTCCCCCATGCTCCTGAGCACCCC  150
GTGGCAACACTCAGCACCATGGAGAGACGCGCCATCAACCTGACCCGGGC  200
25 TAAACCCTTCGACGGCAACAGCCCTCTGATGCGCTACATCTTGAGATGT  250
   CGGAAAACGATGCTCCCTGGACCATACTTCTGGCCAGCGTGACCCAGAAG  300
   CCACCTCCGTGATGGTCAAGGGACTGGTTCCCGCTCGTTCTTACCAGTTC  350
   CGCCTCTGCGCTGTCAACGATGTGGGCAAAGGGCAATTCAGCAAGGACAC  400
   AGAAAGGGTCTCCCTTCCTGAGGAGCCCCCACC GCCCCTCCACAGAACG  450
30 TCATTGCCAGCGGCCGGACCAACCAATCCATCAT  484

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Figure 5. Northern blot analysis of leptin-responsive genes in differentiated PC12-LR8 cells and comparison with the induction in non-differentiated cells.

Adherent PC12-LR8 cells were treated with β -NGF and forskolin for 5 days to induce neuronal differentiation. Differentiated cells were treated with leptin (nF/L) for 24 hours, or were left untreated (nF), in the continued presence of β -NGF and forskolin. Non-differentiated cells were treated for 24 hours with leptin and forskolin (F/L) or with forskolin alone (F). Hybridisation was performed with probes as indicated. Mouse β -actin probe was used as a control. Exposure to BioMax film at -80°C was 3 days for Mx2, 3 hours for peripherin, 4 days for PAP III and 16 hours for β -actin.

Figure 6. Comparison of the induction patterns of different transcripts by leptin and hyper-IL-6 in non-differentiated PC12 cells.

Autoradiographs of Northern blot experiments are shown, using mRNA from PC12-LR8 cells treated with either forskolin, leptin, hyper-IL-6, or combinations thereof.

(A) Induction patterns of Reg I, HIP-I and peripherin after a 12 hours stimulation with forskolin (F), leptin (L), forskolin plus leptin (F/L), hyper-IL-6 (H) or forskolin plus hyper-IL-6 (F/H). Untreated cells were analysed as a control (NI). Exposure times to BioMax MS films for Reg I, HIP-I and β -actin were 4 days, 5 days and 3 hours, respectively; and in a separate experiment for peripherin and β -actin, 16 hours and 2 days.

(B) Induction patterns of PAP I, PAP III, MT-II, Fnk, MRF-1, SOCS-3 and STAT-3 after stimulation with leptin (L) or hyper-IL-6 (H), compared to non-treated control cells (NI). In all cases stimulation was for 12 hours, with the exception of PAP III where stimulation was for 24 hours. β -actin hybridization patterns are shown as control. Exposure times to BioMax MS films at -80°C were 16 hours for PAP I and 1 hour for β -actin; 4 days for PAP III and 1 hour for β -actin; 3 days for MT-II and Fnk, and 2 days for β -actin; 3 days for MRF-1, 2.5 days for SOCS-3 and 2 days for β -actin; and 16 hours for STAT-3 and 3 hours for β -actin.

**Figure 7. DNA sequences of the cloned amplicon corresponding to HIP-I
HIP-I (274 bp)**

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ACAGTTTCTCCTTCCCCAACTTCAGTTCTTCCCTCATTCTTACCCATCCA 50
ATTCTACGCCCCTTATTTCTTGCTCACTTGAAAAACAAAAACACAAACC 100
5 AGATACAACCCTTGCAAAGATATGAAAATTGAAACATAAATATTAAAGCA 150
AATGACCAATGGCAAAGATTGTCAAGATGAGAGAGGAGACATGACAATTG 200
CTTCTCAGTTCCTTGTGTATAGACAATGCCTTATGACATGTGTTTATCAC 250
TCCACTGTAACTAAGATTGTGATT 274

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10

**Figure 8. Kinetics of induction of leptin-responsive genes in non-differentiated
PC12-LR8 cells.**

Autoradiographs of Northern blot experiments using PC12-LR8 cells treated as shown and probed for expression of the indicated genes are shown. T₀ gives the expression level prior to stimulation. Hybridisation with the mouse β -actin probe was used as a control. Sizes of the transcripts are marked on the right.

(A) Northern blot analysis of the immediate early response genes. PC12-LR8 cells were left untreated (NI: non induced), or were treated with forskolin (F), leptin (L) or a combination of both (F/L) for the indicated time points. Exposure times to BioMax MS films for the different transcripts were Fnk: 6 days, MT-II: 5 days, MRF-1: 6 days, SOCS-3: 14 hours, STAT-3: 14 hours, β -actin: 5 hours.

(B) Immediate early response gene-set : early kinetics. PC12-LR8 cells were treated with leptin alone (L) or with a combination of leptin and forskolin (F/L) for the indicated length of time. Exposure time to BioMax MS films were : Fnk: 3 days, MT-II: 3 days, MRF-1: 3 days, SOCS-3: 2.5 days, STAT-3: 2.5 days, β -actin: 1 hour.

(C) Northern blot analysis of the late target genes. PC12-LR8 cells were left untreated (NI), or were treated with forskolin (F), leptin (L) or a combination of both (F/L) for the indicated length of time. Exposure times to BioMax MS films were : Ann VIII: 6 days, PAP I: 14 hours, Squalene Epoxidase: 5 days, UGT: 5 days, β -actin: 14 hours.

30

Figure 9. MT-II, Fnk and PAP I gene expression in leptin treated ob/ob mice.

Leptin deficient ob/ob mice were left untreated (-) or were treated with leptin (100 µg; +) intraperitoneally. Mice were killed five hours after treatment. Northern blot analysis of MT-II and Fnk in liver and PAP I expression in jejunum is shown. Hybridisation with the mouse β-actin probe was used as a control and is shown below. Exposure times to BioMax MS films were 4 hours, 3 days, 4 hours and 8 hours for MT-II, Fnk, PAP I and β-actin respectively.

Figure 10. MT-II and Fnk gene expression in starved wild type mice

Wt mice were starved for 36 hours. After 24 hours mice were treated with PBS (-) or leptin (50 µg, supplemented with 200 µg 2A5 anti human leptin antibody; +). At different time points (-24, 0, 2, 6, 12 hours, indicated on top) mice were sacrificed. RNA was extracted from liver tissue (panel A) or jejunum (panel B) and subjected to Northern blot analysis using MT-II and Fnk as probes as indicated. Hybridisation with the mouse β-actin probe was used as a control and is shown below. Assays were performed and represented in double. Exposure times to BioMax MS films shown in panel A were 2 hours, 2 days and 3 hours for MT-II, Fnk and β-actin respectively. In panel B exposure times were 2 hours for MT-II and 3 hours for β-actin.

Table 1:

Identity of the target gene	Sizes of the different amplicons (bp)	Number of subcloned amplicons	Length of corresponding transcript (kb)	RDA number
Annexin VIII (Ann VIII)	405 327	2 1	2.0	1
Fibroblast Growth Factor-Inducible Kinase (Fnk)	364	3	2.5	1
Hyper-IL-6 Induced Protein (HIP-I)	274	1	1.5	3
Leptin Induced Protein - I (LIP-I)	349	1	6	1
Leptin Induced Protein - II (LIP-II)	484	1	10	1
Metallothionein - II (MT-II)	317	5	0.7	1
Modulator Recognition Factor - 1 (MRF-1)	358	1	2.0	1
Mx2 protein	227	1	2.0	2
Pancreatitis Associated Protein - I (PAP I)	420	2	1.0	1
	462	1		1
	494	2		1
	418	3		3
	496	3		3
Pancreatitis Associated Protein - III (PAP III)	261	8	0.6	2
Peripherin	300	3	1.8	2
Regenerating protein I (Reg I)	214	3	1.2	3
	219	1		
	306	3		
Signal Transducer and Activator of Transcription - 3 (STAT-3)	300	2	4.5	1
	361	2		
Squalene Epoxidase	322	1	3.0	1
Suppressor of Cytokine Signalling-3 (SOCS-3)	389	1	3.0	1
Uridinediphosphate Glucuronyl Transferase (UGT)	361	10	2.5	1

5

Table 1. Characteristics of the identified amplicons. The lengths of the corresponding transcripts were estimated from Northern blot analyses. In the RDA number column, 1, 2 and 3 respectively correspond with an RDA experiment from non-differentiated PC12 cells treated with forskolin or forskolin plus leptin; from differentiated PC12 cells maintained in β -NGF and forskolin and treated with leptin or left untreated; or from non-differentiated PC12 cells treated with hyper-IL-6 or leptin, respectively

10

References

- Baumann, H., Morella, K.K., White, D.W., Dembski, M., Bailon, P.S., Kim, H., Lai, C.F., and Tartaglia, L.A. (1996) The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors. *Proc.Natl.Acad.Sci.U.S.A.*, **93**, 8374-8378.
- Beattie, J.H., Wood, A.M., Newman, A.M., Bremner, I., Choo, K.H., Michalska, A.E., Duncan, J.S., and Trayhurn, P. (1998) Obesity and hyperleptinemia in metallothionein (-I and -II) null mice. *Proc.Natl.Acad.Sci.U.S.A.*, **95**, 358-363.
- Bjorbaek, C., Uotani, S., da Silva, S.B., and Flier, J.S. (1997) Divergent signaling capacities of the long and short isoforms of the leptin receptor. *J.Biol.Chem.*, **272**, 32686-32695.
- Bjorbaek, C., Elmquist, J.K., Frantz, J.D., Shoelson, S.E. and Flier, J.S. (1998) Identification of SOCS-3 as a potential mediator of central leptin resistance. *Molecular Cell*, **1**, 619-625.
- Braun, B.S., Frieden, R., Lessnick, S.L., May, W.A., and Denny, C.T. (1995) Identification of target genes for the Ewing's sarcoma EWS/FLI fusion protein by representational difference analysis. *Mol.Cell Biol.*, **15**, 4623-4630.
- Campfield, L.A., Smith, F.J., Guisez, Y., Devos, R., and Burn, P. (1995) Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks [see comments]. *Science*, **269**, 546-549.
- Chehab, F.F., Lim, M.E., and Lu, R. (1996) Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat.Genet.*, **12**, 318-320.
- Clement, K., Vaisse, C., Lahlou, N., Cabrol, S., Pelloux, V., Cassuto, D., Gormelen, M., Dina, C., Chambaz, J., Lacorte, J.M., Basdevant, A., Bougneres, P., Lebouc, Y., Froguel, P., and Guy-Grand, B. (1998) A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature*, **392**, 398-401.
- Dusetti, N.J., Frigerio, J.-M., Szpirer, C., Dagorn, J.-C. and Iovanna, J.L. (1995) Cloning, expression and chromosomal localization of the rat pancreatitis-associated protein III gene. *Biochem. J.*, **307**, 9-16.
- Flanagan, J.G. and Leder, P. (1990) The kit ligand: a cell surface molecule altered in steel mutant fibroblasts. *Cell*, **63**, 185-194.
- Fischer, M., Goldschmitt, J., Peschel, C., Brakenhoff, J.P., Kallen, K.J., Wollmer, A., Grotzinger, J., Rose-John, S. (1997) A bioactive designer cytokine for human hematopoietic progenitor cell expansion. *Nat. Biotechnol.*, **15**, 142-145.

- Flier, J.S. (1997) Leptin expression and action: new experimental paradigms. *Proc.Natl.Acad.Sci.U.S.A.*, **94**, 4242-4245.
- Flier, J.S. and Maratos-Flier, E. (1998) Obesity and the hypothalamus: novel peptides for new pathways. *Cell*, **92**, 437-440.
- 5 Halaas, J.L., Boozer, C., Blair, W.J., Fidahusein, N., Denton, D.A., and Friedman, J.M. (1997) Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. *Proc.Natl.Acad.Sci.U.S.A.*, **94**, 8878-8883.
- Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D., Lallone, R.L., Burley, S.K., and Friedman, J.M. (1995) Weight-reducing effects of
10 the plasma protein encoded by the obese gene [see comments]. *Science*, **269**, 543-546.
- Holtrich, U., Wolf, G., Brauninger, A., Karn, T., Bohme, B., Rubsamen-Waigmann, H., and Strebhardt, K. (1994) Induction and down-regulation of PLK, a human serine/threonine kinase expressed in proliferating cells and tumors.
15 *Proc.Natl.Acad.Sci.U.S.A.*, **91**, 1736-1740.
- Hubank, M. and Schatz, D.G. (1994) Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic.Acids.Res.*, **22**, 5640-5648.
- Lee, G.H., Proenca, R., Montez, J.M., Carroll, K.M., Darvishzadeh, J.G., Lee, J.I., and Friedman, J.M. (1996) Abnormal splicing of the leptin receptor in diabetic
20 mice. *Nature*, **379**, 632-635.
- Li, B., Ouyang, B., Pan, H., Reissmann, P.T., Slamon, D.J., Arceci, R., Lu, L., and Dai, W. (1996) Prk, a cytokine-inducible human protein serine/threonine kinase whose expression appears to be down-regulated in lung carcinomas. *J.Biol.Chem.*, **271**, 19402-19408.
- 25 Montague, C.T., Farooqi, I.S., Whitehead, J.P., Soos, M.A., Rau, H., Wareham, N.J., Sewter, C.P., Digby, J.E., Mohammed, S.N., Hurst, J.A., Cheetham, C.H., Earley, A.R., Barnett, A.H., Prins, J.B., and O'Rahilly, S. (1997) Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature*, **387**, 903-908.
- 30 Nakamura, Y., Sakakibara, J., Izumi, T., Shibata, A. and Ono, T. (1996) Transcriptional regulation of squalene epoxidase by sterols and inhibitors in HeLa cells. *J.Biol.Chem.*, **271**, 8053-8056.
- Pelleymounter, M.A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T., and Collins, F. (1995) Effects of the obese gene product on body weight regulation
35 in ob/ob mice. *Science*, **269**, 540-543.
- Spiegelman, B.M. and Flier, J.S. (1996) Adipogenesis and obesity: rounding out the big picture. *Cell*, **87**, 377-389.

- Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. (1988) SR alpha promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol.Cell Biol.*, **8**, 466-472.
- Tartaglia, L.A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G.J., Campfield, L.A., Clark, F.T., Deeds, J., and et, a. (1995) Identification and expression cloning of a leptin receptor, OB-R. *Cell*, **83**, 1263-1271.
- 10 Vaisse, C., Halaas, J.L., Horvath, C.M., Darnell-JE, J., Stoffel, M., and Friedman, J.M. (1996) Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. *Nat.Genet.*, **14**, 95-97.
- Verploegen, S.A., Plaetinck, G., Devos, R., Van der Heyden, J. and Guisez, Y. (1997) A human leptin mutant induces weight gain in normal mice. *FEBS letters*, **405**, 237-240.
- 15 Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J.M. (1994) Positional cloning of the mouse obese gene and its human homologue. *Nature*, **372**, 425-432.

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Claims

1. Use of leptin and/or a cytokine which binds to a receptor complex comprising gp130, optionally in combination with a compound acting on adenylate cyclase or
5 acting on one or more of the downstream targets of said cyclase, to activate a signalling cascade whereby as a result thereof immediate early response and/or late target genes are induced in neuro-endocrine cells or in cells from neuro-endocrine origin.
2. Use of leptin and/or a cytokine which binds to a receptor complex comprising
10 gp130 of claim 1, optionally in combination with said compound, wherein the signalling cascade is activated through a leptin receptor.
3. Use of leptin and/or a cytokine which binds to a receptor complex comprising gp130 of claim 1, optionally in combination with said compound, wherein the cells are PC12 cells.
- 15 4. Use of leptin and/or a cytokine which binds to a receptor complex comprising gp130 of claim 1, optionally in combination with said compound, wherein the compound is forskolin.
5. Use of leptin and/or a cytokine which binds to a receptor complex comprising gp130 of claim 1, optionally in combination with said compound, wherein the
20 immediate early response genes are STAT-3, SOCS-3, Metallothionein-II, the serine/threonine kinase Fnk and the rat homologue of MRF-1 and the like and wherein the late target genes are Pancreatitis-Associated Protein I, Squalene Epoxidase, Uridinediphosphate Glucuronyl Transferase, Annexin VIII, Leptin Induced Protein I and II (LIP I, II), Regenerating Protein I, Hyper-IL-6 Induced
25 Protein (HIP-I), Pancreatitis-Associated Protein III, Mx2 or Peripherin.
6. Method of screening for molecules which interfere directly or indirectly with the induction of immediate early response genes and/or late target genes or with the activity of the products of said genes of claim 1, wherein said genes are inducible by leptin and/or a cytokine which binds to a receptor complex comprising gp130,
30 optionally in combination with a compound acting on adenylate cyclase or acting directly or indirectly on one or more of the downstream targets of said cyclase.

7. Molecules obtainable by the screenings method of claim 6.
8. Pharmaceutical composition comprising a molecule according to claim 7.

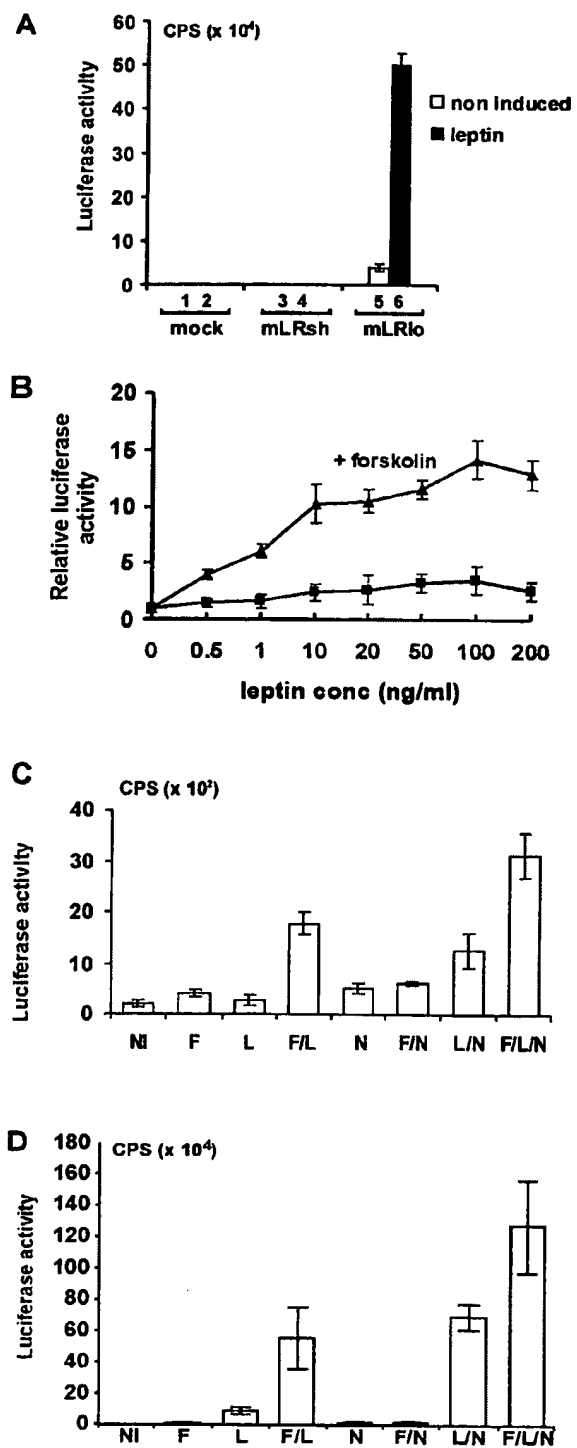


Figure 1

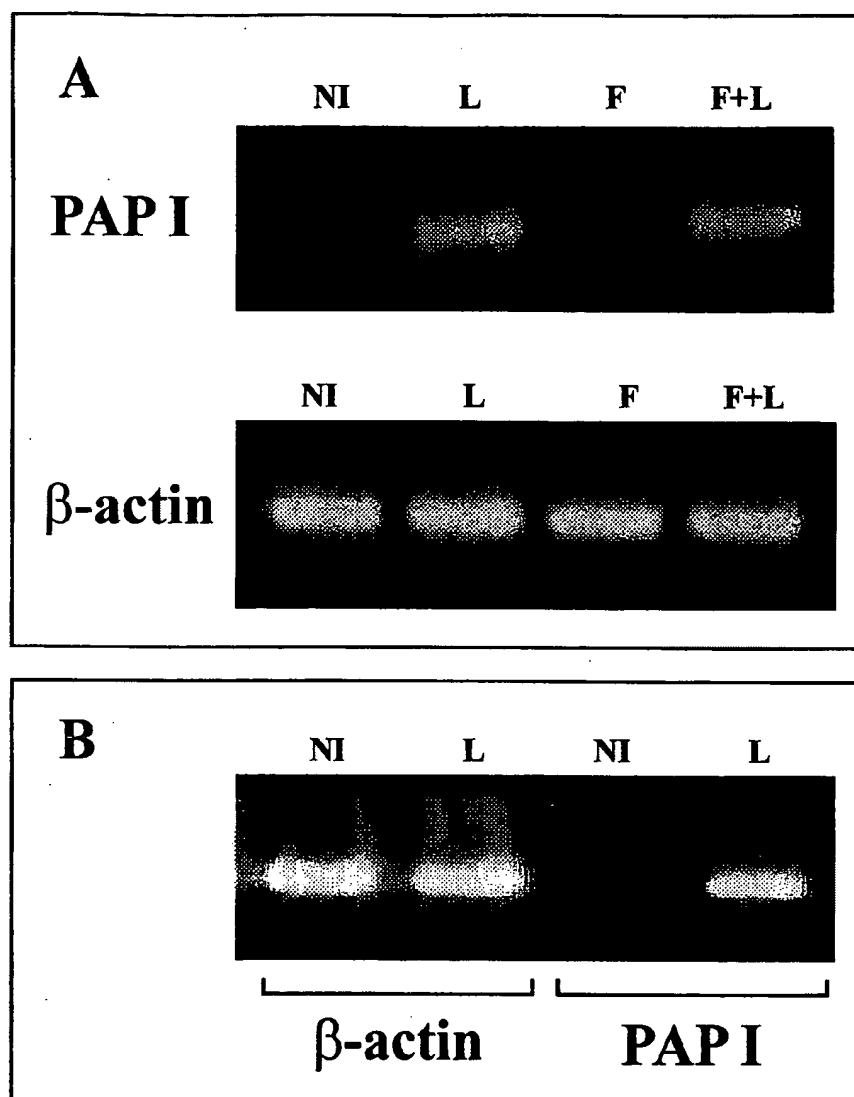


Figure 2

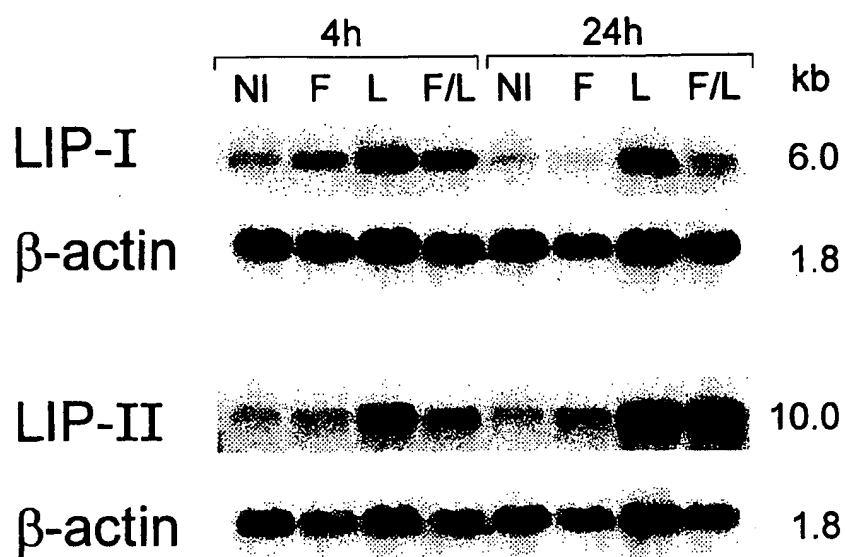


Figure 3

Figure 4.**LIP-I (349bp)**

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GTCTCGGTCCAAGGCCTCTGCCCAGGCCAGCTGTCCTGTGGCAAGGTGG 150
GTGGAGTGGAAACTGCTTCCTGTCTGCTGGGCCAGAGTCTCTTCATG 200
CCGGACTCAGAAACCAGCTACATCCTGAGCTGTGGTGTTCAGGTCTCCA 250
10 GGGCAAGGCACCACCGAAGCGCAATGGCACCAGCTCCAGTGTGGGGCCCCG 300
GCTGCTCAGATGCCCCCACCACCCCATCAGACAGAAGGCCCGCTTCAA 349

LIP-II (484bp)

15 CGCCTGGACAGAAATGGCTCCCTACACATCTCGCAGACATGGTCAGGGGA 50
CATTGGCACGTATACCTGCCGGGTACTCTCAGCTGGTGGCAATGACTCTC 100
GCAACGCCCACCTGCGAGTCAGGCAGCTCCCCCATGCTCCTGAGCACCCC 150
GTGGCAACACTCAGCACCATGGAGAGACGCGCCATCAACCTGACCCGGGC 200
TAAACCCTTCGACGGCAACAGCCCTCTGATGCGCTACATCTTGGAGATGT 250
20 CGGAAAACGATGCTCCCTGGACCATACTTCTGGCCAGCGTGACCCAGAAG 300
CCACCTCCGTGATGGTCAAGGGACTGGTTCCCGCTCGTTCTTACCAGTTC 350
CGCCTCTGCGCTGTCAACGATGTGGGCAAAGGGCAATTGAGCAAGGACAC 400
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25

30

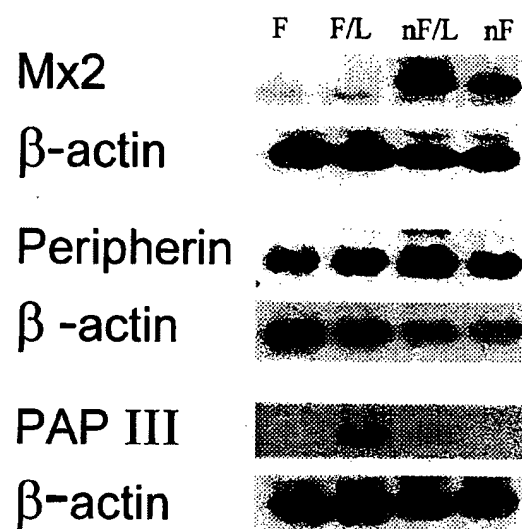


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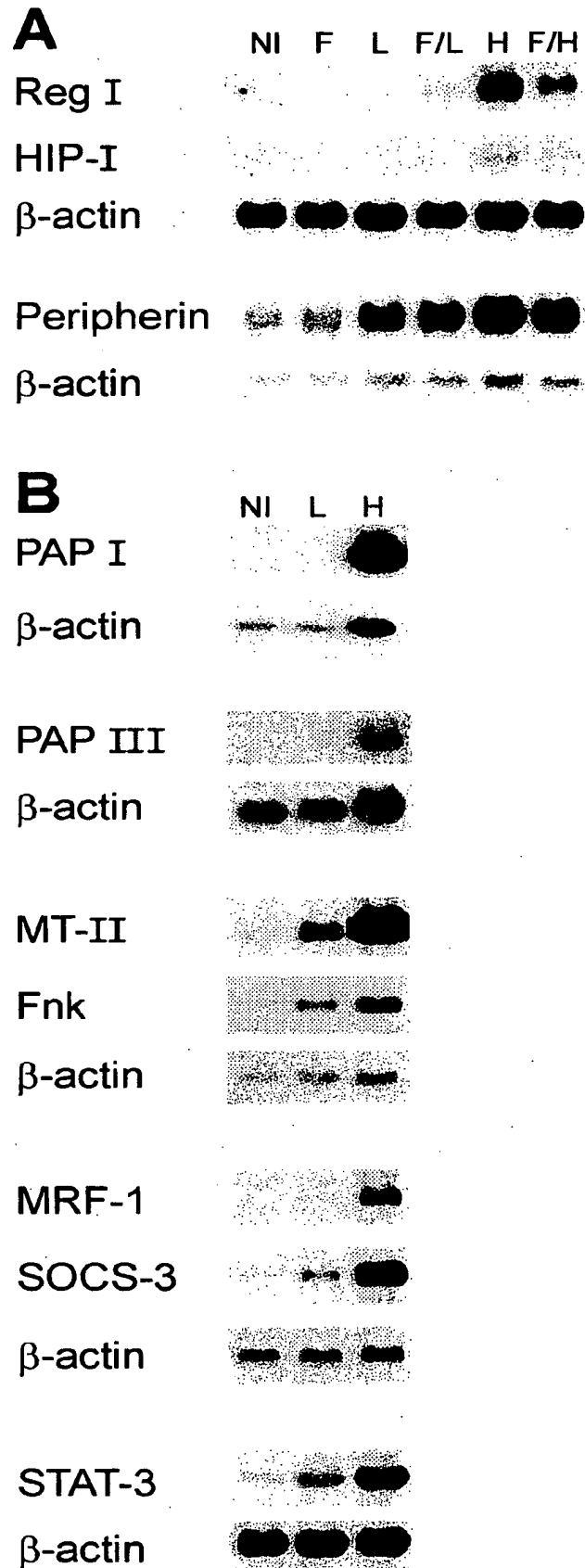


Figure 6

Figure 7.

5 **HIP-I (274 bp)**
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AGATAACAACCCTTGCAAAGATATGAAAATTGAAACATAAATATTAAAGCA 150
AATGACCAATGGCAAAGATTGTCAAGATGAGAGAGGAGACATGACAATTG 200
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TCCACTGTAACTAAGATTGTGATT 274

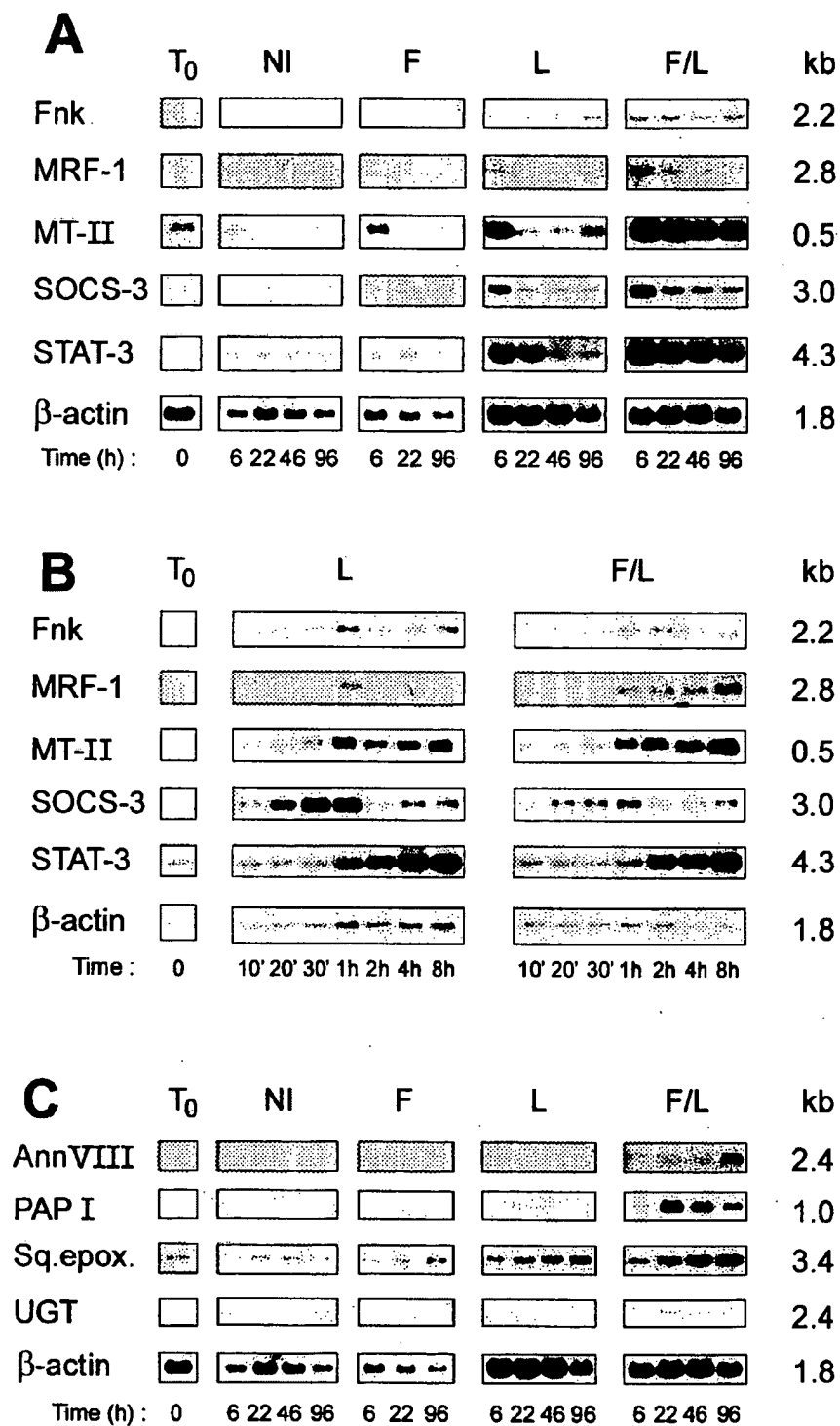


Figure 8

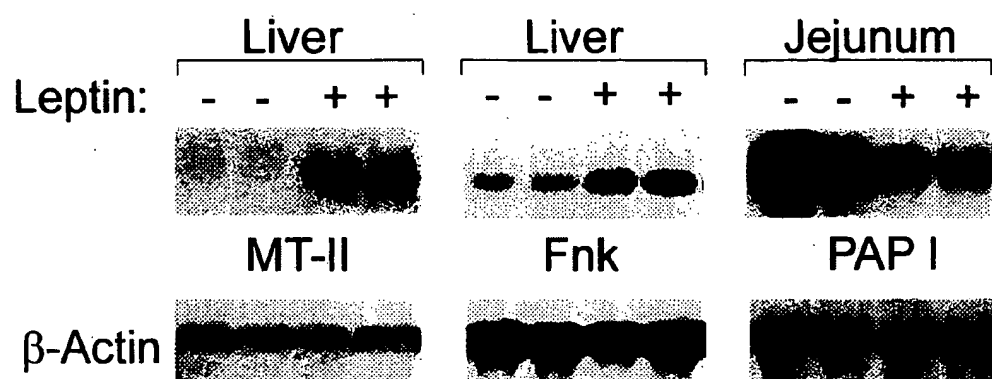


Figure 9

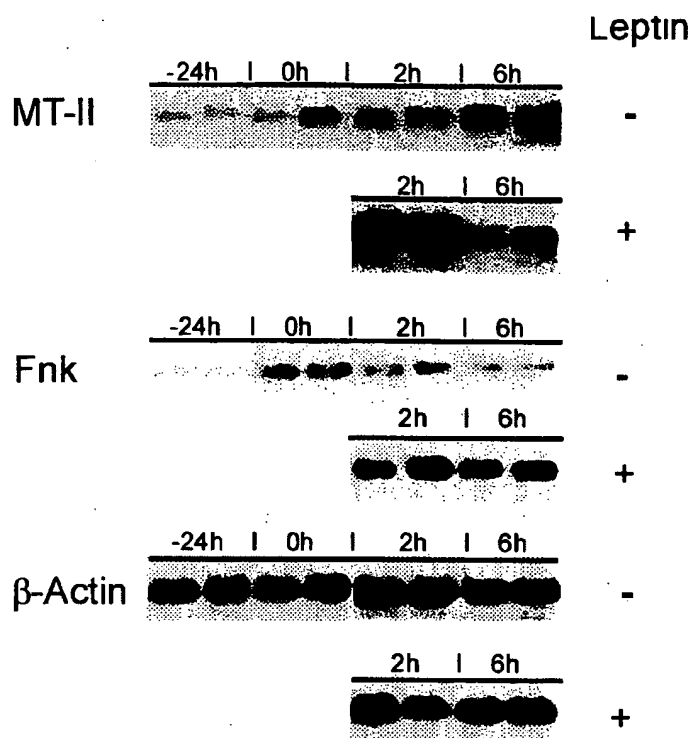
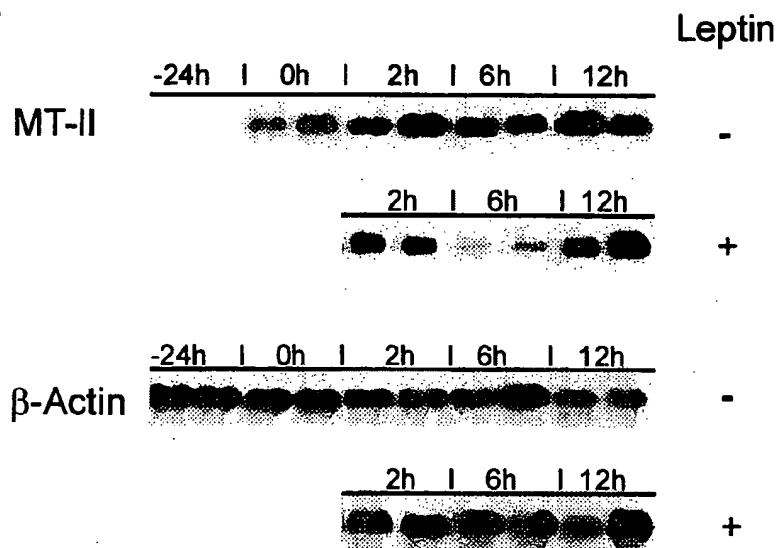
A**B**

Figure 10

SEQUENCE LISTING

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caggtctcca gggcaaggca ccaccgaagc gcaatggcac cagctccagt gtggggcccg 300
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tatgaaaatt gaaacataaa tattaagca aatgaccaat ggcaaagatt gtcaagatga 180
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